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Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development

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Abstract

Recent studies of mouse mutant *aphakia* have implicated the homeobox gene *Pitx3* in the survival of substantia nigra dopaminergic neurons, the degeneration of which causes Parkinson's disease. To directly investigate a role for *Pitx3* in midbrain DA neuron development, we have analysed a line of *Pitx3*-null mice that also carry an eGFP reporter under the control of the endogenous *Pitx3* promoter. We show that the lack of Pitx3 resulted in a loss of nascent substantia nigra dopaminergic neurons at the beginning of their final differentiation. *Pitx3* deficiency also caused a loss of tyrosine hydroxylase (TH) expression specifically in the substantia nigra neurons. Therefore, our study provides the first direct evidence that the *aphakia* allele of *Pitx3* is a hypomorph and that Pitx3 is required for the regulation of TH expression in midbrain dopaminergic neurons as well as the generation and/or maintenance of these cells. Furthermore, using the targeted GFP reporter as a midbrain dopaminergic lineage marker, we have identified previously unrecognised ontogenetically distinct subpopulations of dopaminergic cells within the ventral midbrain based on their temporal and topographical expression of Pitx3 and TH. Such an expression pattern may provide the molecular basis for the specific dependence of substantia nigra DA neurons on Pitx3.

Keywords: Differentiation; Dopaminergic neuron; Embryonic stem cell; GFP; Homeobox; Knock-in; *Pitx3*; Transcription factor; Tyrosine hydroxylase

Introduction

The midbrain dopaminergic (DA) neurons are located in the ventral midbrain and form the substantia nigra pars compacta (SNc/A9) and the ventral tegmental area (VTA/A10) (Bjorklund and Lindvall, 1984). Dopaminergic neurons of the SNc regulate motor function via nigro-striatal projections to the dorsolateral striatum, and it is these neurons that preferentially degenerate in Parkinson's disease. Dopaminergic neurons of the VTA, on the other hand, project to the ventromedial striatum and cortical areas, forming the mesolimbocortical system, which is involved in mood and reward behaviour. Defects in this system are implicated in schizophrenia and drug abuse (Hermanson et al., 2003).

The correct specification and development of the midbrain DA neurons depends on the proper development of the midbrain/hindbrain boundary and the expression of several signalling molecules and transcription factors including *En-1*, *En-2*, *Pax2*, *Pax5* and *Wnt1* (McMahon and Bradley, 1990; Schwarz et al., 1997; Simon et al., 2001; Ye et al., 1998). Other factors critical for midbrain DA neuron development include Shh and FGF8, the combined activity of which defines the position of dopaminergic precursors in the ventral mesencephalon (Ye et al., 1998). The orphan nuclear hormone receptor *Nurr1*, which is required for dopamine neurotransmitter phenotype, is detected in the ventral midbrain at around E10.5 in mice just before the expression of the key enzyme tyrosine hydroxylase (TH) at E11 (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1996).

Within the central nervous system, the paired-like homeobox protein Pitx3 is expressed exclusively in DA

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neurons of the SNc and the VTA (Smidt et al., 2004, 1997; Zhao et al., 2004). Midbrain expression of *Pitx3* is first seen at E11.5 and is maintained throughout life in both rodents and humans (Smidt et al., 1997). In *aphakia* mutant mice, in which *Pitx3* expression is hypomorphic due to deletions of the presumed *Pitx3* promoter region and non-coding exon 1, the midbrain DA neurons are formed initially, but the SNc neurons are lost later in foetal development (Hwang et al., 2003; Nunes et al., 2003; Rieger et al., 2001; Semina et al., 2000; Smidt et al., 2004; Van Den Munckhof et al., 2003). Questions remain, however, as to the exact developmental stage at which Pitx3 is required during midbrain DA development and why SNc DA neurons are preferentially affected even though Pitx3 is expressed in all DA neurons of the SNc and VTA (Smidt et al., 2004; Zhao et al., 2004).

We have generated ES cells and mice in which the entire *Pitx3* coding sequence is replaced with an eGFP reporter via homologous recombination (Zhao et al., 2004). Quantitative studies demonstrated that *Pitx3*-GFP reporter is expressed in all SNc and VTA DA neurons in the adult midbrain (Zhao et al., 2004). Furthermore, this *Pitx3*-GFP knock-in allowed the genetic labelling of ES-cell-derived midbrain DA cells. In this study, we have investigated the function of Pitx3 in midbrain DA neuron differentiation via two complementary approaches: (i) a developmental study of the midbrain DA system in *Pitx3*-null mice and (ii) in vitro dopaminergic differentiation of ES cells overexpressing Pitx3.

Materials and methods

Plasmid construction

The *Pitx3* cDNA was amplified by RT-PCR using E14.5 brain RNA, sequence verified and cloned into the dicistronic pPyCAG-IP vector, downstream of the constitutive expression unit CAG (Chambers et al., 2003). The puromycin resistant gene (*pac*) is linked downstream of the *Pitx3* cDNA via an internal ribosome entry site (IRES) to ensure that all puromycin resistant cells co-express the *Pitx3* cDNA.

ES cell culture and transfection

ES cells were maintained in GMEM supplemented with 2-mercaptoethanol, non-essential amino acids, sodium bicarbonate, 10% foetal calf serum (FCS) and 100 units/ml LIF on gelatinised tissue culture flasks (Smith, 1991). A *Pitx3*-GFP ES cell line, PTG2, was used for expressing *Pitx3* transgene (Zhao et al., 2004). For transfection with the Pitx3 expression construct and vector control DNA, 2×10^7 PTG2 ES cells were electroporated with 10 μg of linearised plasmid DNA at 800 V and 3 μF in a 0.4-cm cuvette using a Bio-Rad gene pulser. Transfected ES cells were selected in the presence of 1.5 μg/ml puromycin (Sigma). 12 independent Pitx3 clones and 4 mock control clones were isolated. Puromycin selection was applied in routine ES cell culture to ensure maintenance of transgene expression. The Pitx3 overexpressing ES cells will be referred to as Pitx3 ES cells.

In vitro differentiation

In vitro differentiation of ES cells on PA6 stromal cells was carried out as previously described (Kawasaki et al., 2000). Briefly, parental PTG2 and Pitx3 overexpressing PPT ES cells were cultured on a layer of PA6 stromal cells for 7 days in GMEM supplemented with knock-out serum replacement at 70 cells per cm^2. From day 7, the above medium was replaced with DMEM/F12 supplemented with N2 (Gibco) and ascorbic. Medium was refreshed every other day, and cultures were terminated at day 14 and processed for immunostaining. Three independent Pitx3 lines (Pitx3-1.-2 and-4) and two control lines were tested with similar results. Most of the experiments, however, were carried out using Pitx3-1, Pitx3-2 and one of the control ES lines.

Immunostaining

Cultures were washed twice in phosphate-buffered saline (PBS) then fixed in 4% paraformaldehyde for 20 min. Brains were fixed by immersion in 4% paraformaldehyde overnight (embryo) or for 4 days (adult), cryoprotected with 30% sucrose, sectioned at 30 μm on a cryostat and collected in PBS or directly on slides. Sections to be visualised with DAB were incubated in 3% H_2O_2 in methanol for 15 min at 4°C to quench endogenous peroxidase activity. Fixed cells and floating sections were then blocked with 3% normal serum, 1% BSA and 0.2% Triton X-100 in PBS and were incubated with primary antibodies (Pitx3 1:500, rabbit, gift from Dr. Marten Smidt, TH, 1:1000, rabbit, Pel Freeze; En1, 1:200, mouse, Developmental Studies Hybridoma Bank, University of Iowa; DAT, 1:500, rabbit, Chemicon; GFP, chicken, 1:2500, Chemicon; βTubulin3 (TuJ1), 1:500, mouse, Babco; Ki67, 1:500, rabbit, Novocastra; GAD, 1:3000, rabbit, Sigma) in blocking solution at 4°C overnight. Cells/sections were washed three times for 20 min each in PBS with 1% BSA and 0.2% Triton X-100 then incubated with fluorescence-labelled secondary antibodies for 1 h at room temperature (overnight at 4°C for sections). After washing three times with PBS, cell/sections were mounted in Immunofluore (ICN Biomedicals) and analysed using a Zeiss Axiophot microscope or Leica confocal microscope using a Zeiss Axiophot microscope or Leica confocal microscope.

TUNEL labelling

Cryostat sections were processed as described for immunostaining with anti-GFP antibody then processed according to manufacturers instructions using ApopTag Red In Situ Apoptosis Detection Kit (Chemicon).

Nissl staining

Coronal wax sections (10 μm) from newborn midbrain of *Pitx3* heterozygous and *Pitx3* homozygous mice were Nissl-stained in a solution containing 1% cresyl violet acetate (Sigma), acidified with 0.25% glacial acetic acid.

Quantitative analysis of immunolabelled cells

To determine the total number of immunopositive cells present in the midbrain, all labelled cells were counted in serial coronal sections (30 μm), and the number of cells from each section was summed. Total counts from four–five E12.5 mice of each genotype (*Pitx3* wt, *Pitx3* heterozygous and *Pitx3* homozygous) were averaged, and standard deviations were calculated. On average, a TH/GFP labelled mesencephalon from an E12.5 brain spanned 10 sections. Total counts of DA neurons of E14.5 midbrains were obtained using this approach. At E14.5, some sections crossing the rostral and caudal part of midbrain do not exhibit typical SNc and VTA architecture. This caused difficulties in terms of categorising labelled cells into either the SNc or VTA group accordingly for all sections. Therefore, to determine the relative number of DA neurons present in either the SNc or VTA, we carried out comparative counts by examining 5 typical sections per animal for each genotype (three animals per genotype, Fig. 4). Statistical analysis for the counting data was performed using two samples Student's *t* test.

To determine the number of antibody-labelled cells in differentiated ES cell cultures, all positively stained cells in a well from a four-well plate were counted. In instances where neurons were too numerous to count accurately (>500, Fig. 8), a count of 500 was assigned. Antibody staining was performed in duplicates, and the data obtained from at least two independent experiments were averaged.

Results

Identification of two subgroups of midbrain DA neurons by temporal and topographical differential expression of TH and Pitx3

While it has been established that the midbrain expression of *Pitx3* RNA starts at E11.5 in mice (Smidt et al., 1997), it is not clear whether Pitx3 is expressed in dopaminergic precursors or in differentiated DA neurons. Therefore, we sought to examine Pitx3 expression in direct comparison with TH throughout the developing midbrain between E12.5 and E14.5. We first compared the expression of the GFP reporter with that of Pitx3 protein by antibody staining in phenotypically normal heterozygous *Pitx3* mice (Table 1 and Zhao et al., 2004). As shown in Fig. 1, at E12.5, all GFP expressing cells were labelled with an anti-Pitx3 antibody. This result indicates that, similar to the adult midbrain, the *Pitx3*-GFP reporter faithfully mirrors Pitx3 protein expression (Smidt et al., 2004; Zhao et al., 2004). Therefore, in the following studies, we used the knock-in GFP reporter to mark Pitx3 expressing cells. We started with E12.5 midbrain as this is the earliest developmental stage at which a GFP signal could be visualised.

At the most rostral level of E12.5 mesencephalon, the GFP^+ and TH^+ domains lie at comparable dorsal–ventral levels with the GFP^+ cells located lateral to TH^+ cells (Figs. 2A–B). Moving caudally from the rostral part of the mesencephalon, the GFP^+ and TH^+ domains partially overlap with each other with the TH expressing field positioned relatively dorsally and the GFP^+ domain lying ventrally and laterally (Figs. 2C–F). Consequently, there are a significant number of GFP^+TH^- cells in the ventrolateral area in the rostral part of the mesencephalon, with GFP^-TH^+ cells lying dorsomedial to them (Figs. 2C–F). Towards the caudal part of the E12.5 mesencephalon, the GFP^+ and TH^+ domains

Table 1
Quantitative analysis of E12.5 and E14.5 mesencephalic DA cells in *Pitx3* mutant mice

Age	Genotype	GFP^+TH^-	GFP^+TH^+	Total TH^+
E12.5	*Pitx3* wt (n = 4)	n/a	n/a	1870 ± 293
	Pitx3+/– (n = 5)	607 ± 378	1210 ± 316	1814 ± 469
	Pitx3–/– (n = 5)	809 ± 264	628 ± 362	834 ± 419
	$P\leq$	0.35[a]	0.029[a]	0.84[b]
E14.5 total	*Pitx3*+/– (n = 3)	1834 ± 341	17622 ± 4397	
	Pitx3–/– (n = 3)	6773 ± 1356	14175 ± 3600	
	$P\leq$	0.004	1	
E14.5 SNc	*Pitx3*+/– (n = 5)	189 ± 7.9	1415 ± 43.7	
	Pitx3–/– (n = 5)	519 ± 15.3	596 ± 11.3	
	$P\leq$	2.733×10^{-5}	4.363×10^{-5}	
E14.5 VTA	*Pitx3*+/– (n = 5)	272 ± 29.8	1161 ± 25.3	
	Pitx3–/– (n = 5)	150 ± 16.1	813 ± 19.9	
	$P\leq$	0.2698	0.003417	

Embryo heads were fixed by immersion in 4% paraformaldehyde overnight, cryoprotected with 30% sucrose, sectioned coronally at 30 μm on a cryostat and collected in PBS. Sections were double stained with anti-GFP and anti-TH antibodies. All labelled cells were counted in serial sections, and the numbers of cells from each section were summed. E14.5 SN and VTA results were obtained from 5 sample midbrain sections from each genotype ([a] Student's *t* test between *Pitx3*+/– and *Pitx3*–/–; [b] Student's *t* test between *Pitx3* wt and *Pitx3*–/–; n/a, not applicable).

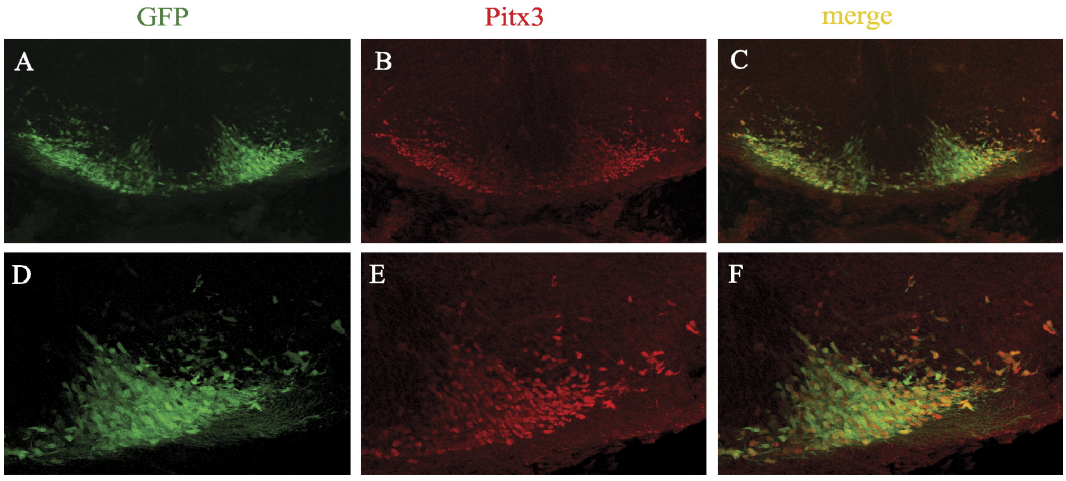

Fig. 1. *Pitx3*-GFP reporter mirrors Pitx3 protein expression in developing mesencephalic cells. (A–F) Coronal sections of E12.5 midbrain were double labelled with antibodies against GFP and Pitx3, ×20 magnification (A–C) and ×40 magnification (D–F).

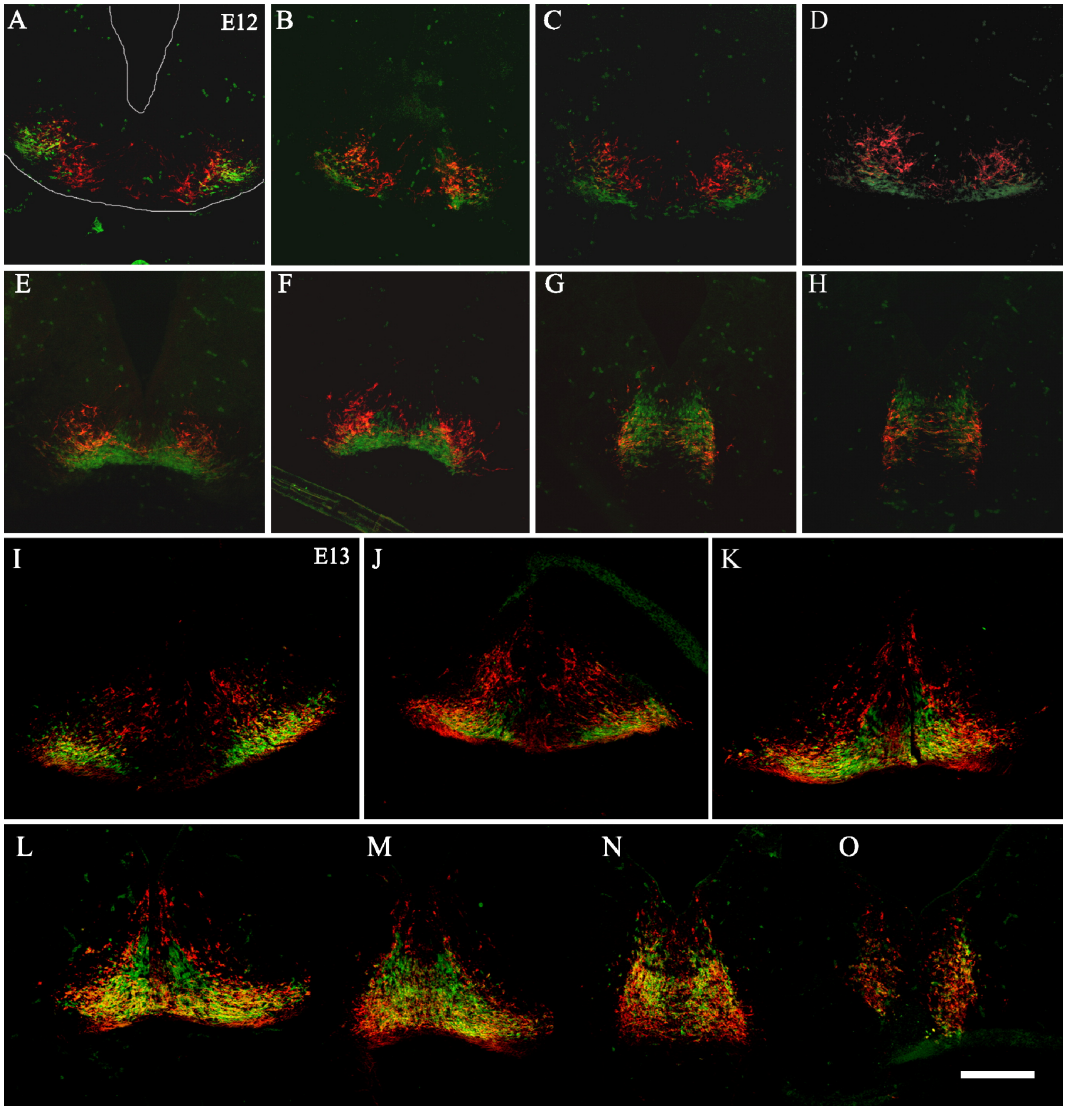

Fig. 2. Differential temporal and topographical expression of Pitx3 defines subregional heterogeneity within the mesencephalic DA lineage. (A–O) Coronal sections of heterozygous E12.5 (A–H) and E13.5 (I–O) mesencephalon were double labelled with anti-TH (red) and anti-GFP (green) antibodies. Section series are shown from rostral to caudal. Scale bars: 200 μm.

merge together with most of the cells located dorsomedially (Figs. 2G, H). The shape of the GFP^+TH^+ domain at this location is suggestive of a developing VTA.

Looking closely at the orientation of the cells in the midbrain DA neuron domain at E12.5 (Supplementary Fig. 1A) reveals the apparent migration pattern of the midbrain DA neurons and progenitors. Cells located medially have a vertical orientation, suggesting that they are migrating vertically ventralwards from the neuroepithelium (Supplementary Fig. 1B). Whereas, the cells in the lateral location have a horizontal orientation, indicating that they are migrating horizontally lateralwards towards their final destination (Supplementary Fig. 1C).

The presence of distinct GFP^+ and TH^+ domains is less pronounced at E13.5, as a significant proportion of cells are now double positive, suggesting that the cells that were single positive at E12.5 have now gained expression of either GFP or TH (Figs. 2I–O). This was particularly apparent in the caudal part of the E13.5 midbrain (Figs. 2M–O). By E14.5, apart from a small number of cells in rostral lateral SNc primordium that expressed GFP alone, most of the cells were labelled with both GFP and TH (Figs. 3C–C″ and Zhao et al., 2004). In the adult midbrain, all GFP^+ cells were also TH^+ and vice versa (Figs. 6G, H; Zhao et al., 2004).

To investigate the cellular identity and developmental status of the GFP^+TH^- cells at E12.5, we have examined the expression of markers for mitotic progenitors (Ki67), midbrain dopaminergic neurons and their progenitors Engrailed 1 (En1), GABAergic neurons (GAD), nascent neurons (βTubulin3) and glial cells (S100β and GFAP). We found that the majority of *Pitx3*-GFP^+ cells express En1, whilst only a proportion of the En1-expressing cells express *Pitx3*-GFP. Most of the *Pitx3*-GFP^+ cells, except few positioned medially, express Tuj1. However, none of the

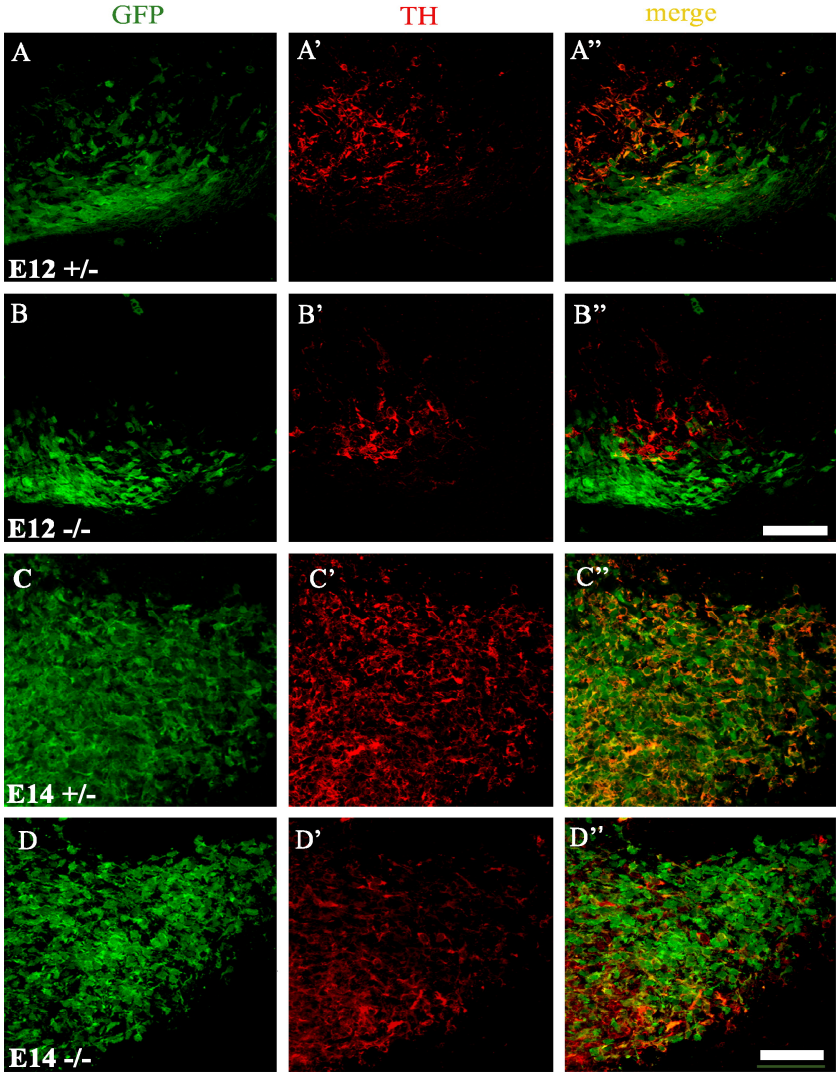

Fig. 3. Analysis of Pitx3 and TH expression in the developing mesencephalon of *Pitx3*-null mice. (A–B) TH and GFP double stained E12.5 coronal sections show a reduced number of TH^+ and GFP^+ cells in *Pitx3*−/− (B–B″) as compared to *Pitx3*+/− (A–A″) mesencephalon. (C–D) TH and GFP double labelling on E14.5 sections showing an increase of GFP^+TH^- cells in the SNc of *Pitx3*−/− mice (D–D″) as compared to *Pitx3*+/− mice (C–C″). Scale bar: 50 μm for panels (A) and (B), 200 μm for panels (C) and (D).

Pitx3-GFP$^+$ cells stained positive for Ki67, GAD, S100β and GFAP (Supplementary Fig. 2 and data not shown). Therefore, our data suggest that the E12.5 GFP$^+$ cells are mostly postmitotic neurons.

Taking these expression studies together, our data indicate that mesencephalic DA neurons and their immediate progenitors are subdivided into two partially overlapping groups based on their temporal expression profile of TH and Pitx3: the ventrolateral mesencephalic cells express Pitx3 prior to TH, whilst the dorsomedial midbrain DA neurons express TH ahead of Pitx3.

Pitx3 is required during the transition of postmitotic mesencephalic DA progenitors to TH$^+$ neurons

The above finding prompted us to investigate in detail the developmental fate of different subgroups of midbrain cells in *Pitx3*-null mice. Firstly, we established, by counting TH$^+$ cells in the entire E12.5 mesencephalon, that the heterozygous mutation of *Pitx3* does not affect mesencephalic DA development as these mice contained a similar number of TH$^+$ neurons as compared to the *Pitx3* wild type littermates (Table 1). Therefore, we used *Pitx3* heterozygous mice as controls in the following studies so that the GFP reporter could be exploited to track the fate of Pitx3 expressing neurons in *Pitx3*-null mice. Serial sections of the entire E12.5 and E14.5 midbrains were double stained with antibodies against TH and GFP, and the number of TH$^+$ and GFP$^+$ cells was analysed quantitatively. At E12.5, we found no statistical difference in the number of GFP$^+$TH$^-$ cells between *Pitx3* homozygous mutants and *Pitx3* heterozygous controls (Table 1), suggesting that the generation and the maintenance of midbrain DA progenitor neurons are not affected by Pitx3 deficiency even though Pitx3 is normally expressed in these cells. However, a 50% and 45% reduction in the number of GFP$^+$TH$^+$ and total TH$^+$ cells, respectively, was observed in E12.5 homozygous *Pitx3* mutants (Table 1, Figs. 3A–B″). A significant reduction in the number of TH$^+$ DA neurons at this developmental stage has not been previously reported in *aphakia* studies (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). Therefore, our data demonstrate directly and for the first time that Pitx3 is essential for the formation of the full complement of nascent DA neurons and that the *aphakia* allele of *Pitx3* is a hypomorph.

At E14.5, it was evident that the loss of DA neurons was more severe in the forming SNc than in the VTA (Figs. 3C–D″). This observation was confirmed by a comparative quantitative analysis carried out separately in the two regions (Table 1, Fig. 4A).

To analyse whether *Pitx3*-null GFP$^+$ neurons die by apoptosis, we performed a combined TUNEL assay with GFP antibody staining in E14.5 *Pitx3*-null and *Pitx3* heterozygous control mice (n = 3). This study revealed that the *Pitx3*-deficient mice have a significantly increased number of apoptotic cells in the SNc as compared to *Pitx3* heterozygous mice. However, no difference was detected in the VTA region (Fig. 5A).

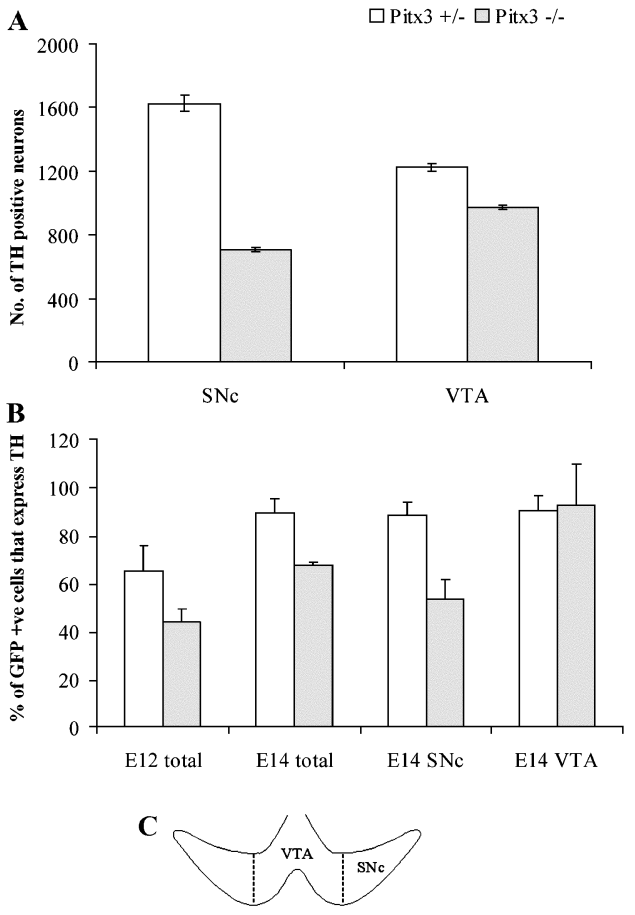

Fig. 4. Pitx3 regulates TH expression specifically in the SNc DA neurons. (A) Quantitative data demonstrate a preferential reduction in the number of TH$^+$ cells in the E14.5 SNc of *Pitx3*−/− mice. (B) Quantitative analyses showing reductions in the percentage of TH expressing cells in the GFP$^+$ population of E12.5 and E14.5 *Pitx3*−/− midbrain. (C) Diagram illustrating how the SNc and VTA are defined in a typical E14.5 midbrain section. Data were obtained by examining five typical midbrain sections from three *Pitx3*+/− and three *Pitx3*−/− mice.

TH expression in the substantia nigra DA neurons requires Pitx3

Previous gel shift and transient transfection experiments demonstrated that Pitx3 can activate the *TH* promoter via a high-affinity binding site (Cazorla et al., 2000; Lebel et al., 2001). Our finding that Pitx3 is expressed prior to TH in some ventral mesencephalic cells during development points to a potential role for Pitx3 as a physiological activator for *TH* expression. We found that, at E12.5, the proportion of TH$^+$ cells within the GFP$^+$ population was already significantly reduced in the *Pitx3* knockout mesencephalon (43.7 ± 5.5%) as compared to their heterozygous littermates (64.8 ± 10.6%) (P <= 0.03, Fig. 4A). Therefore, our data uncover a new function of Pitx3 in regulation of TH expression in a subset of developing midbrain DA cells.

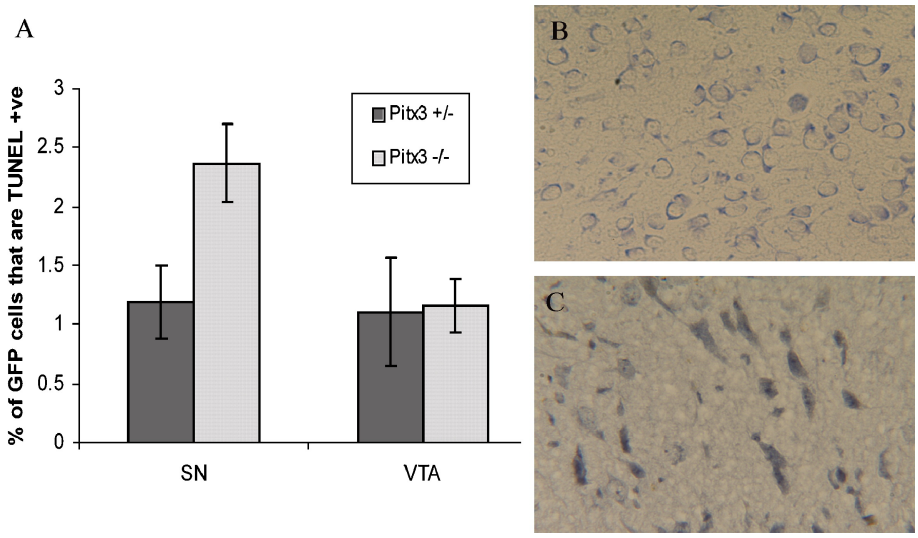

Fig. 5. TUNEL analysis of developing *Pitx3*+/− and *Pitx3*-null mice. (A) Percentage of TUNEL labelled GFP-expressing cells in the SNc and VTA. E14.5 midbrain sections from *Pitx3*+/− and *Pitx3*−/− mice (3 mice were used for each genotype) were processed for TUNEL assay using protocols suggested by the manufacture. There are significantly more TUNEL labelled GFP^+ cells in the SNc of *Pitx3*−/− mice (2.34 ± 0.33%) as compared to the SNc of *Pitx3*+/− mice (1.18 ± 0.31%), $P \leq 0.01$. In contrast, there is little difference between the number of TUNEL labelled GFP^+ cells in the VTA of *Pitx3*−/− mice (1.16 ± 0.23%) and the VTA of *Pitx3*+/− mice (1.11 ± 0.46%), $P \leq 0.85$. (B–C) Nissl-stained coronal midbrain sections at the level of the SNc from *Pitx3*+/− (B) and *Pitx3*−/− (C) newborn mice, ×40 magnification.

The finding that some *Pitx3*-null mesencephalic progenitor neurons still acquire TH expression establishes that the regulation of TH expression by Pitx3 is not required by all midbrain DA neurons and that the necessity for this Pitx3 function may display subregional specificity. If this is the case, we would expect to see restricted loss of TH in particular subregions of the mutant midbrain. To investigate this possibility, we examined GFP and TH double stained midbrain sections at E14.5 when SNc and VTA primordium becomes recognisable (Figs. 3C, D). Specific loss of TH expression was visible in the SNc in homozygous *Pitx3* mutant midbrain when compared to heterozygous littermates (Figs. 3C, D and 4B). This was accompanied by a considerable loss of GFP^+TH^+ neurons in the *Pitx3* knockout SNc (Table 1, Fig. 4A). In the SNc, however, the reduction in the number of TH^+ cells was greater than that of GFP^+ cells, as evidenced by the presence of a greater number of GFP^+TH^- neurons in the *Pitx3*-null mice when compared to the *Pitx3* heterozygous controls (Figs. 3D–D″ and Table 1). This was also reflected by a reduction in the percentage of TH^+ neurons in the GFP^+ population in *Pitx3* homozygous mutant SNc (53.4%) as compared to *Pitx3* heterozygotes (88.2%, Fig. 4A). These data demonstrate that two elements contributed to the reduction of TH expressing cells in E14.5 *Pitx3* knockout SNc: (1) an absence of midbrain DA neurons (i.e. the loss of GFP^+TH^+ cells) and (2) a failure of TH expression in remaining GFP^+ neurons. Since GFP^+TH^- neurons were confined to the forming SNc, our data suggest that Pitx3 is required specifically by the SNc GFP^+ neurons for initiation and/or maintenance of TH expression.

Midbrain DA deficiency in the adult Pitx3-null mice

To investigate whether Pitx3 is continuously required in the adult midbrain DA system, we performed immunostaining on adult brain sections at the level of midbrain and striatum with antibodies against TH. Similar to results obtained with the E12.5 mesencephalon, we found no difference in the number or distribution of TH^+ cells between the heterozygous and the wild type mice (data not shown). However, a marked reduction in TH-positive neurons was again observed primarily in the SNc of the homozygous *Pitx3* mutant mice as compared to the wild type and *Pitx3* heterozygous mutants (Fig. 6 and data not shown). The loss of TH^+ cells appears to be more severe in adult than in E14.5 midbrain (Figs. 3D–D″, 6C, D). In contrast to findings in the E14.5 midbrains, the loss of TH expression in the adult midbrain was closely mirrored by a loss of GFP expression in *Pitx3*-deficient SNc. However, GFP was still expressed in DA neurons of VTA in *Pitx3*-deficient mice, although a reduction in the number of cells was apparent as compared to the heterozygous control mice (Fig. 6). Thus, the absence of GFP^+ cells in the SNc of *Pitx3*-deficient mice suggests that midbrain DA neurons are absent rather than phenotypically defective in the homozygous *Pitx3* mutant mice. Therefore, our findings on the relative expression patterns of *Pitx3*-GFP and TH in foetal and adult midbrain brains suggest that the loss of SNc DA neurons occurred progressively, starting from the beginning of DA terminal differentiation into adulthood. At least for some SNc DA neurons, down regulation of TH expression took place first and cell death occurred later. This is supported by the finding that the number of

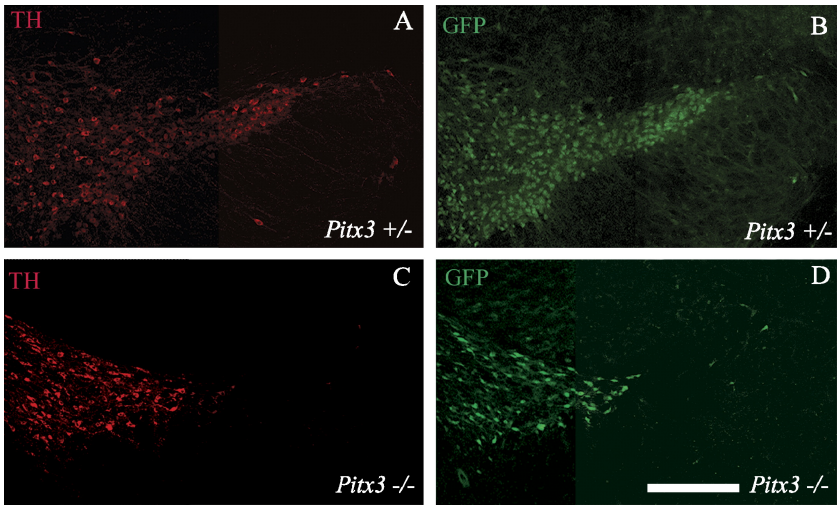

Fig. 6. Continued requirement for Pitx3 in the adult midbrain DA system. Immunohistochemistry for TH and *Pitx3*-GFP was performed on adult brain sections of *Pitx3*+/− (A–B) and *Pitx3*−/− (C–D) mice showing the loss of both TH and GFP expressing cells in the mutant brain. Scale bar: 300 μm.

apoptotic cells, as detected by TUNEL and Nissl staining, increases in *Pitx3*-null midbrain at E14.5 and in neonates, respectively (Fig. 5). Furthermore, Nissl staining has shown that there are fewer neurons in *Pitx3* homozygous SNc and that many of these cells have pyknotic nuclei as compared to *Pitx3* heterozygous mice (Fig. 5B, C).

Overexpression of Pitx3 in ES cells promotes the generation of midbrain specific DA neurons

Our analysis of the *Pitx3*-null mice demonstrates a key role for this molecule in the early processes of postmitotic differentiation of midbrain DA neurons. To further investigate the functional capacity of Pitx3, we have carried out a gain of function study in ES cells. We have shown previously that *Pitx3*-GFP$^+$ neurons derived from *Pitx3*-GFP knock-in ES cells express DA markers and behave similarly to primary midbrain DA neurons with respect to trophic factor and neural toxin responsiveness (Zhao et al., 2004). Therefore, we engineered the *Pitx3*-GFP knock-in ES cells to constitutively express *Pitx3* transgene (Pitx3 ES cells) and asked whether overexpression of Pitx3 has an effect on DA neuron production in vitro (Fig. 7A).

In the presence of LIF, Pitx3 ES cells behaved similarly to control ES cells in terms of proliferation rate and expression of the stem cell marker Oct4 (data not shown). They do not express *Pitx3*-GFP or TH at undifferentiated state. ES cells from two independent clones of Pitx3 transfectants were induced for DA neuronal differentiation using PA6 bone marrow-derived stromal cells (Kawasaki et al., 2000). In this system, ES-cell-derived *Pitx3*-GFP$^+$ cells can be readily detected at days 13–14 (Zhao et al., 2004). These cells appeared in clusters with various numbers of GFP$^+$ cells which often formed part of a larger group of TH$^+$ cells in a differentiated ES cell colony (Zhao et al., 2004). Not all TH$^+$ clusters contained GFP$^+$ cells however.

To ask whether Pitx3 overexpression promotes a midbrain DA fate, we examined the expression of midbrain and general DA markers including *Pitx3*-GFP, En1, TH and DAT by antibody staining at 14 days of differentiation. Quantitative analysis revealed that the differentiating Pitx3 ES cell cultures contained around 5 times more GFP$^+$ and 3 times more GFP$^+$TH$^+$ or GFP$^+$DAT$^+$ cells, respectively, than in control cultures (Figs. 7B, C, 8A). However, no significant change was observed in the total number of DA neurons (i.e. TH$^+$ or DAT$^+$ cells) in Pitx3 overexpression cultures (Fig 7A), rather, there was in increases in the proportion of TH$^+$ and DAT$^+$ cells that also express midbrain marker *Pitx3*-GFP in Pitx3 cultures (Fig. 8B). Furthermore, we found that, similar to control cultures, the majority of the GFP$^+$ cells in Pitx3 cultures were also En1$^+$ (control: 94.3 ± 3.8%; Pitx3: 91.4 ± 4.1%, Figs. 7G–I, 8C). These data suggest that the increased production of GFP$^+$ cells resulting from Pitx3 overexpression reflects a true induction of midbrain cell identity.

The expression of Pitx3 in postmitotic midbrain DA progenitors suggests that Pitx3 is more likely to influence the terminal differentiation and/or survival of DA neurons rather than the initial generation of ventral neural precursors. We tested this hypothesis by examining the number of En1$^+$ neural progenitor cells produced in Pitx3 and control ES cultures. Since En1 is expressed in early midbrain neural precursors as well as in differentiated DA neurons (Alberi et al., 2004; Joyner and Martin, 1987), we performed our study at day 8 of in vitro differentiation when the production of neural precursor cells peaks and *Pitx3*-GFP$^+$ neurons could not be detected. This experiment revealed a similar number of En1$^+$ cells in the Pitx3 (9.2 ± 1.1%) and the control (10.3 ± 2.5%) cultures. In addition, we examined the frequency of *Pitx3*-GFP$^+$ clusters at day 14 and again found no difference in the percentage of differentiating ES cell colonies that contain

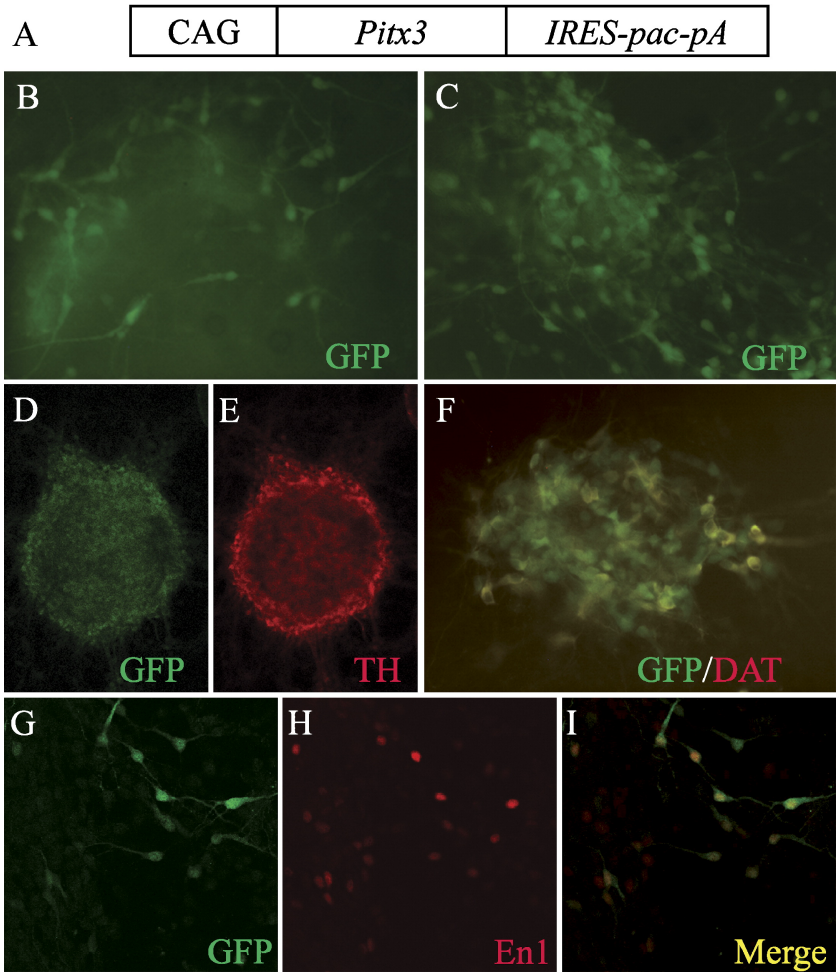

Fig. 7. Dopaminergic differentiation of ES cells forced expressing Pitx3. ES cells were co-cultured with PA6 stromal cells for 14 days followed by direct visualisation of the *Pitx3*-GFP (B, C) and double immunostaining for GFP/TH (D, E), GFP/DAT (F) and GFP/En1 (G–I). The control cultures (B) produced fewer GFP^+ cells than in Pitx3 ES cell cultures (C). Shown in panels (D) and (E) are differentiated Pitx3 ES cell colonies that consist primarily of GFP^+TH^+ cells. (F–I) ES-cell-derived $Pitx3^+$ cells co-express other DA (DAT) or midbrain (En1) markers.

either *Pitx3*-GFP^+ or TH^+ clusters between the Pitx3 (47.2 ± 9.8% and 70.5 ± 12% respectively) or the control cultures (45.7 ± 19% and 67 ± 11.5%, respectively). Therefore, our data suggest that Pitx3 does not appear to have an effect on the generation of mesencephalic dopaminergic precursors.

Nurr1 has been shown to promote a DA fate from ES cells in a neuronal independent fashion (Sonntag et al., 2004). This prompted us to investigate whether the induction of dopaminergic features by Pitx3 is neuronal dependent. We analysed *Pitx3*-GFP and TH expression in conjunction with a pan-neuronal marker βTubulin3 and an astrocyte marker GFAP. The majority of the *Pitx3*-GFP^+ cells in differentiated Pitx3 (97.5 ± 7%) and the control (98.7 ± 8%) cultures expressed neuronal marker βTubulin3. Those few $\beta Tubulin3^-$*Pitx3*-GFP^+TH^- cells exhibit a large flat morphology, and they do not express other neural markers such as Nestin (data not shown). Furthermore, no *Pitx3*-GFP^+ cells were found to be $GFAP^+$ in either the control or Pitx3 cultures. Therefore, the data demonstrate that Pitx3 induces dopaminergic phenotype in the context of neuronal fate.

Discussion

Phenotypic analysis of the *Pitx3*-null mice generated by gene targeting and overexpression of *Pitx3* transgene in ES cells were carried out to gain insights into the functional requirement for the transcription factor Pitx3 in midbrain DA neuron development. We present the first evidence that Pitx3 is required for the expression of TH in the SNc, as well as for the generation and/or maintenance of nascent midbrain DA neurons. Our study provides phenotypic evidence that the *aphakia* allele of *Pitx3* is a hypomorph. Furthermore, we have identified ontogenetically distinct subpopulations of DA cells within the ventral midbrain based on their differential temporal and topographical expression of TH and Pitx3 by exploiting the targeted GFP reporter. This finding may provide a potential rational

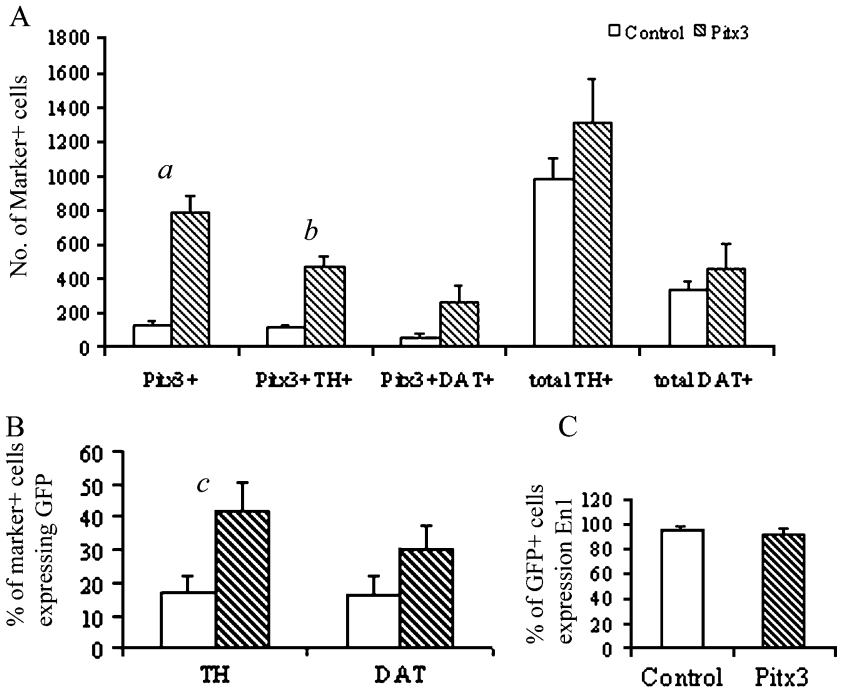

Fig. 8. Quantitative analysis of neuronal marker expression by Pitx3 overexpressing ES cells. (A) Average number of cells per well that were labelled with *Pitx3*-GFP, TH or DAT. Note that there was no significant difference in the total number of TH^+ or DAT^+ cells. (B) The above data are presented as percentage of TH^+ or DAT^+ cells that co-express *Pitx3*-GFP. (C) Quantitative analysis for the proportion of cells expressing midbrain marker En1. Data shown are average of three independent experiments for panels (A) and (B) and two experiments for panel (C). Experiments were performed with two independent Pitx3 clones for panels (A) and (B) and a single Pitx3 clone for data obtained in panel (C). Two sample Student's *t* test was performed to compare the control and Pitx3 overexpressing cultures. Statistical significance found was marked as *a*–*c*. The *P* values (≤) are: 0.013 (*a*), 0.021 (*b*) and 0.04 (*c*), respectively.

for the substantia nigra specific DA neuronal defects displayed in the *Pitx3*-null mice.

Requirement for Pitx3 in terminal differentiation of the SNc DA neurons

The current study establishes that Pitx3 is essential for the transition stage from postmitotic DA progenitors (TH^-) to TH^+ DA neurons based on the finding that *Pitx3*-GFP^+TH^+ but not *Pitx3*-GFP^+TH^- cells are lost in *Pitx3*-null mutants at E12.5. This observation supports the notion that Pitx3 is dispensable for the specification of postmitotic DA progenitor neurons.

The classical mouse mutation *aphakia* affects the presumed *Pitx3* regulatory elements whilst leaving the entire coding sequences intact (Fig. 9B; (Rieger et al., 2001; Semina et al., 2000). Consequently, the *aphakia* mice have 5% of the wild type level of *Pitx3* transcript from E11 to newborn (Rieger et al., 2001). Although Pitx3 protein in midbrain DA neurons was reported to be undetectable using immunohistochemical methods (Smidt et al., 2004; Van Den Munckhof et al., 2003), the possibility that low amounts of protein are present cannot be excluded. Therefore, it is not clear whether this mutation represents a true null or whether its phenotype fully reflects the requirement for Pitx3 in midbrain development. In order to address this question, we generated mice with a complete deletion of the *Pitx3* coding sequence. We found that the *Pitx3* knockout caused more severe midbrain DA defects than those observed in *aphakia*. For example, other studies reported normal density of TH expressing cells in E12.5 *aphakia* mice (Smidt et al., 2004; Van Den Munckhof et al., 2003), whilst we see an over 50% reduction in the number of TH expressing cells in our *Pitx3* homozygous mutant mice. Furthermore, our study revealed a requirement for Pitx3 in regulating Th expression in SNc DA neurons.

A preferential loss (>50%) of SNc DA neurons was reported in neonatal and adult *Tgfα* mutant mice (Blum, 1998). Could there be any link between the Pitx3 and Tgfα? At birth and in the adult, the *Pitx3*-null and the *aphakia* mice suffer from a more dramatic cell loss than the *Tgfα* mutants, suggesting that Pitx3 is unlikely to act downstream of *Tgfα*.

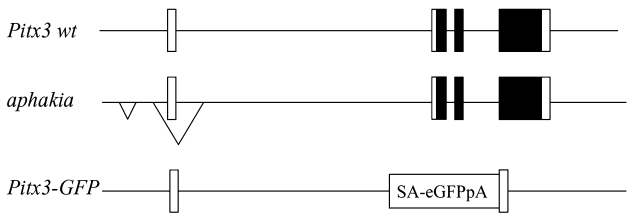

Fig. 9. Schematic illustration showing the genomic structure of the *Pitx3* wild type, *aphakia* and the *Pitx3*-GFP targeted locus. The *Pitx3* gene contains four exons. The coding regions are indicated with filed boxes. The two deletions previously identified in *aphakia* are marked by triangles (drawing not to scale).

Conversely, we have found by RT-PCR that the expression of *Tgfα* is not down regulated in FACS purified *Pitx3*-null midbrain DA neurons as compared to the *Pitx3* heterozygous cells (Vives and Li, unpublished). This observation suggests that *Tgfα* does not lie downstream of Pitx3, although it may be possible that Pitx3 is able to potentiate *Tgfα* activity during midbrain DA neuron development.

Pitx3 as a physiological regulator for TH expression

A potential role for Pitx3 in the induction of Th expression has been suggested by in vitro promoter studies (Cazorla et al., 2000; Lebel et al., 2001). Our finding that a subset of *Pitx3*-deficient midbrain DA cells lack TH expression during development provides the first demonstration that Pitx3 is a physiological regulator for TH. It is interesting that the regulation of TH by Pitx3 is region dependent: the SNc was affected, whereas the VTA remained relatively normal. Given that neurons of the SNc and VTA differ in their target innervation and physiological functions, it is conceivable that TH expression is critically regulated by alternative mechanisms in different DA neuronal subtypes.

Nurr1 serves as a general TH regulator within the midbrain. TH expression is lost in both the SNc and VTA DA neurons in *Nurr1* mutants. The present study indicates that TH regulation in SNc DA neurons also involves the Pitx3 pathway. Pitx3 may exert this regulatory role independently or via regulating or interacting with Nurr1. Transcriptional regulation of *Nurr1* by Pitx3 may be unlikely as *Nurr1* RNA was detected in FACS-purified E12.5 and E14.5 *Pitx3*-deficient midbrain DA cells, although a region specific down regulation of *Nurr1* cannot be excluded (Vives and Li, unpublished observation). Pitx3 and Nurr1 can co-operate to activate Th promoter in vitro (Cazorla et al., 2000). Therefore, Pitx3 may interact with Nurr1 in regulating TH expression in the SNc. In either case, the SNc specific dependence of midbrain DA neurons on Pitx3 may be explained by the temporal and subregional expression profile of Pitx3 during development.

Members of the Pitx homeodomain protein family have been shown to interact with bHLH transcription factors and T box proteins to achieve cell type-specific transcription regulation of hormone genes in pituitary development (Lamolet et al., 2001; Poulin et al., 2000). In sympathetic neurons, the homeobox transcription factors Phox2a and Phox2b regulate neurotransmitter phenotype by activating TH and β-hydroxylase expression through interaction with bHLH proteins (Morin et al., 1997; Pattyn et al., 1997). Therefore, one could hypothesise that Pitx3 regulates TH in the ventral midbrain via interactions with as yet unidentified, perhaps SNc specific regulator(s).

Actions of Pitx3 on embryonic stem cell differentiation

Our loss of function studies of the physiological requirement for Pitx3 in midbrain DA neuron differentiation, survival and TH regulation were complemented by gain of function studies in ES cells. Overexpression of Pitx3 in ES cells did not have a significant effect on the total number of TH^+ cells produced, rather, it resulted in a significant increase in the proportion of TH^+ cells expressing midbrain marker (*Pitx3*-GFP). Significantly, the majority of ES-cell-derived GFP^+ cells also express an independent midbrain marker En1, suggesting that overexpression of Pitx3 promotes the production of a true midbrain DA phenotype rather than merely auto-regulating *Pitx3* expression. Our finding is in keeping with a recent report where transgene expression of Pitx3 in ES cells resulted in a specific increase in the number of DA neurons that express AHD2, a marker that is expressed in ventral midbrain dopaminergic neurons as well as in their precursors (Chung et al., 2005; Wallen et al., 1999). We also show here that overexpression of Pitx3 did not affect the proportion of $En1^+$ midbrain neural progenitors generated during differentiation. Therefore, our data suggest that the action of transgenic Pitx3 is primarily to

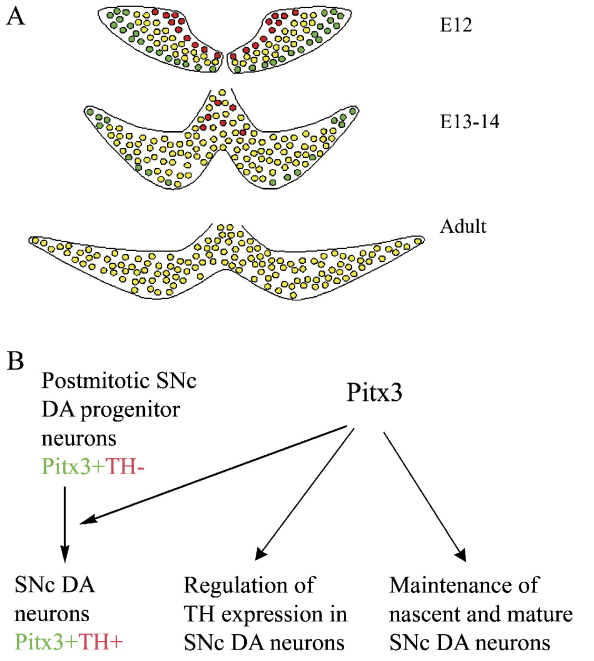

Fig. 10. Schematic illustration of the ontogeny of midbrain DA neurons and function of Pitx3 in these cells. (A) Diagrams summarising the expression of Pitx3 and TH during development from E12.5 to adulthood. At E12.5, cells that only express TH (red) lie in a dorsal/medial position and the cells that express Pitx3 (green) alone locate in a lateral/ventral position within the midbrain DA primordium. These two subgroups overlap where cells are both TH and Pitx3 positive (yellow). By E13.5 and E14.5, most midbrain DA cells express both TH and Pitx3 except some ventral lateral most cells that are $Pitx3^+TH^-$. Dorsally, some cells are TH^+ only. In the adult VTA and SNc, all midbrain DA neurons co-express Pitx3 and TH (Zhao et al., 2004). (B) Summary of the functions of Pitx3 in midbrain DA neuron development. Pitx3 is essential for the generation and/or maintenance of nascent midbrain DA neurons at early steps of terminal differentiation, as well as the regulation of TH expression specifically in SNc DA neurons. Furthermore, Pitx3 is continuously required for the survival of midbrain DA neurons in late foetal development and in adult life.

promote the acquisition of a midbrain fate from DA precursors and is less likely to be involved in the induction of an early ventral mesencephalic fate from stem cells. Pitx3 may also act to enhance survival of differentiated DA neurons in our ES cell system.

Unlike Nurr1, which appears to promote DA phenotype in both neuronal and non-neuronal cells (Bjorklund et al., 2002; Chung et al., 2002; Kim et al., 2003a,b; Sonntag et al., 2004; Wagner et al., 1999), DA differentiation facilitated by Pitx3 overexpression is confined to neuronal cells. Our study suggests that Pitx3 has the ability to promote midbrain DA fate within the context of a competent (TH^+) cell population. Therefore, overexpression of Pitx3 may provide a potential paradigm for generating physiologically relevant DA neurons for use in biopharmaceutical screening and cell therapy applications.

Ontogenetic distinction between the midbrain DA cells and its relation with SNc specific Pitx3-dependence

In this study, we discovered that the midbrain DA neurons arise from two subpopulations of cells as marked by their differential expression profile of TH and Pitx3 (Fig. 10). This developmental subregional heterogeneity suggests that midbrain DA neuron development may proceed via both Pitx3-dependent and Pitx3-independent mechanisms, and this distinction in ontogeny is reflected in the selective vulnerability of the SNc DA lineage in *Pitx3* mutants. Several lines of evidence suggest that the early (E12.5) $Pitx3^+TH^-$ cells contribute to histogenesis of the SNc: (1) $Pitx3^+TH^-$ cells are already located lateral to the TH^+ domain of cells in the rostral most mesencephalon at E12.5 (Figs. 2A–H, 10A). This is in keeping with the fact that the SNc DA neurons are organised in more lateral and rostral regions relative to that of VTA. (2) At E12.5, TH^+ cells closer to the medial part of the neuroepithelium exhibit a vertical orientation, whereas cells close to the apical surface of the ventral midbrain have a more horizontal orientation. This is in agreement with previous finding that DA neurons migrate firstly ventralwards and subsequently lateral and rostrally towards their definite site in SNc, which occurs between E11 and E16 in mice (Kawano et al., 1995). (3) The $Pitx3^+TH^-$ cells were found primarily in the primordium of SNc but not VTA of E14.5 midbrain in both the *Pitx3* heterozygous and *Pitx3* homozygous mice (Figs. 3C–D″). (4) Finally, defective DA neuron development occurred preferentially in the SNc resulting in the specific loss of DA neurons in the adult SNc.

This model differs from that proposed by Van Den Munckhof et al. (2003), suggesting that the SNc specific loss of DA neurons in later foetal development of *aphakia* mice is due to the differential expression of Pitx3 in ventral part of the SNc, an observation that was neither supported by our study nor by Smidt et al. (2004) and Zhao et al. (2004). Our model proposes that the specific dependence of substantia nigra DA neurons on Pitx3 is controlled by developmentally regulated differential expression of Pitx3 within a subgroup of otherwise phenotypically similar (i.e. dopaminergic) neurons.

In summary, the current work establishes that Pitx3 is essential for the birth and/or survival of a subset of nascent DA neurons at E12.5, as well as TH expression and/or maintenance of the SNc DA neurons in later foetal development. Pitx3 does not appear to be required for the migration of SNc DA progenitors, however, as $Pitx3^+TH^-$ neurons were found in the SNc of E14.5 *Pitx3*−/− midbrain. The *Pitx3* knockout mice, together with *Pitx3*-GFP ES cells, provide good model systems for future investigations into genetic programs and molecular mechanisms controlling specification and maintenance of midbrain specific DA neurons, the cell type that relates to Parkinson's disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.03.028.

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